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INTRODUCTION

c-Myc is a bHLH-ZIP transcription factor that regulates the expression of a large number of target genes, which collectively promote transformation. The active form of c-Myc exists as a heterodimer with another bHLH-ZIP protein, Max. This interaction, along with c-Myc-Max sequence-specific DNA binding ability is necessary for all of c-Myc's biological properties, including transformation.

The factors which make c-Myc a compelling therapeutic target have recently been reviewed (1). Also addressed were the various levels at which pharmacologic attacks upon c-Myc, other members of the c-Myc network, or c-Myc target genes might be aimed. Among the strategies thus far employed (with limited success) are the targeting of the *CMYC* gene with triplex-forming oligonucleotides, the use of anti-sense oligonucleotides to target c-Myc mRNA, the use of short, double-stranded E-box containing oligonucleotides that serve as "decoy' binding sites, and the use of dominant-negative forms of c-Myc. The strengths and limitations of these approaches were discussed at length in this review.

Using a yeast two-hybrid-based approach in which the interaction between the bHLH-ZIP domains of c-Myc and Max can be easily quantified, we have previously identified low molecular weight compounds which disrupt this interaction (2). It was subsequently shown that these compounds could inhibit DNA binding by c-Myc-Max heterodimers and could inhibit the growth of c-Myc-transformed cells. The major problem with these problems is their relatively low potencies, with significant inhibition of tumor cell growth being obtained only at 50-100 μM. For this reason, their clinical application is likely to be limited.

As a potential solution to the above problem, the laboratory has embarked upon a project designed to identify more potent analogs (3). This objective was considerably helped by our recent determination that most, if not all of the compounds bound to the c-Myc monomer and thus did not require the presence of Max (see below).

The long-term objective of the laboratory is to solve the 3D solution structure of these so-called "parental compounds" in association with the c-Myc monomer. In this way, we will be able to utilize structure-based computational approaches in the rational design of compounds with greater potencies.

ORIGINAL SPECIFIC AIMS OF THIS APPLICATION

***Task 1*: To demonstrate that each of these compounds either prevents or disrupt c-Myc-Max heterodimerization.**

***Task 2*: To conduct a series of *in vivo* studies aimed as determining whether these compounds can be effectively employed to treat c-Myc overexpressing tumors.**

***Task 3*: To determine whether any of the compounds can be utilized in combination as a means of reducing toxicity and potentiating the antineoplastic effect.**

***Task 4*: To employ computerized "data mining" techniques to identify the structural properties of these compounds which impart their effectiveness.**

ACCOMPLISHMENTS

A. Task 1: To demonstrate that each of these compounds either prevents or disrupt c-Myc-Max heterodimerization.

1. 10058-F4 and its analogs bind directly to c-Myc.

10058-F4 is one of the original "parental compounds" that we first identified (2). We have determined the activities of a large number of analogs of 10058-F4 and have identified a number with potencies 5-10 fold greater than 10058-F4 itself (REF. 3) The studies described below were undertaken to determine precisely how these compounds affected the c-Myc-Max association.

We took advantage of the fact that 10058-F4, and most of its analogs, are fluorescent and can depolarize light. Because this property is partly an inverse function of the molecule's rate of tumbling in solution, binding to either c-Myc or Max should result in a loss of fluorescence depolarization. This occurred in the case of 10058-F4 and all active analogs upon the addition of c-Myc$_{353-439}$, an 86 amino acid recombinant form of the bHLH-ZIP dimerization domain of human c-Myc. In contrast, inactive 10058-F4 analogs failed to bind (Fig. 1). Similar experiments performed with recombinant Max protein failed to provide evidence for binding of any of the compounds (not shown). These results support the idea that the activities of 10058-F4 and its analogs arise from their ability to bind directly to the c-Myc bHLH-ZIP monomer.

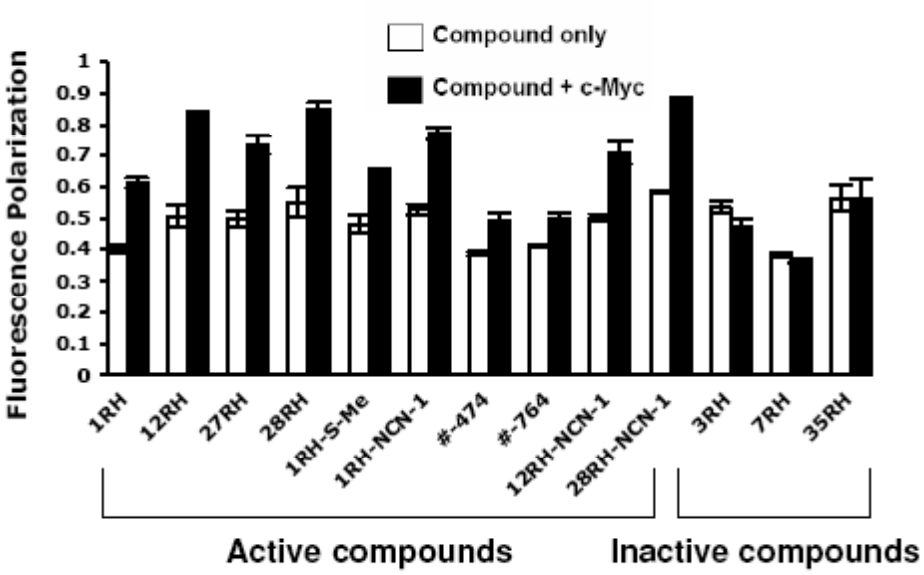

Figure 1. Fluorescence polarization assays. Each of the indicated compounds was assayed in triplicate at a final concentration of 25 µM in the presence or absence of 25 µM recombinant c-Myc$_{353-439}$. Excitation and emission maxima were 380 nM and 468 nM, respectively. For structures of all 10058-F4 derivative please see the attached re-print (REF. 3).

2. Identification of the binding site for 10058-F4.

Having determined that the c-Myc bHLH-ZIP monomer is sufficient for 10058-F4 binding, we next asked if we could better define its binding site. Thus, we expressed and purified to homogeneity a series of overlapping N- and C-terminal bHLH-ZIP deletions and used these in the

above-described fluorescence polarization assay. These studies (Fig. 2) confirm that 10058-F4 does indeed bind to the c-Myc bHLH-ZIP domain, and implies this site to center around amino acids 400-409.

We further confirmed the findings with 10058-F4 in two ways. First, we generated >100 full-length, His6-tagged c-Myc bHLH domain proteins with random amino acid mutations and sequenced each one. We chose several double and triple point mutations that fell within the suspected binding region for 10058-F4 and tested these by fluorescence polarization. These results (Fig. 3) were in complete agreement with those obtained with the larger deletions, thus localizing the site of binding to amino acids ca. 400-410. Second, we determined whether the suspected region of binding was sufficient for 10058-F4 binding. To this end, we synthesized the peptide (Y402ILSVQAEEQK412) and used it for circular dichroism (CD) studies. As shown in Fig. 4, the addition of 10058-F4 induced a dramatic change in α-helical content of the peptide and once again confirmed direct binding to monomeric c-Myc.

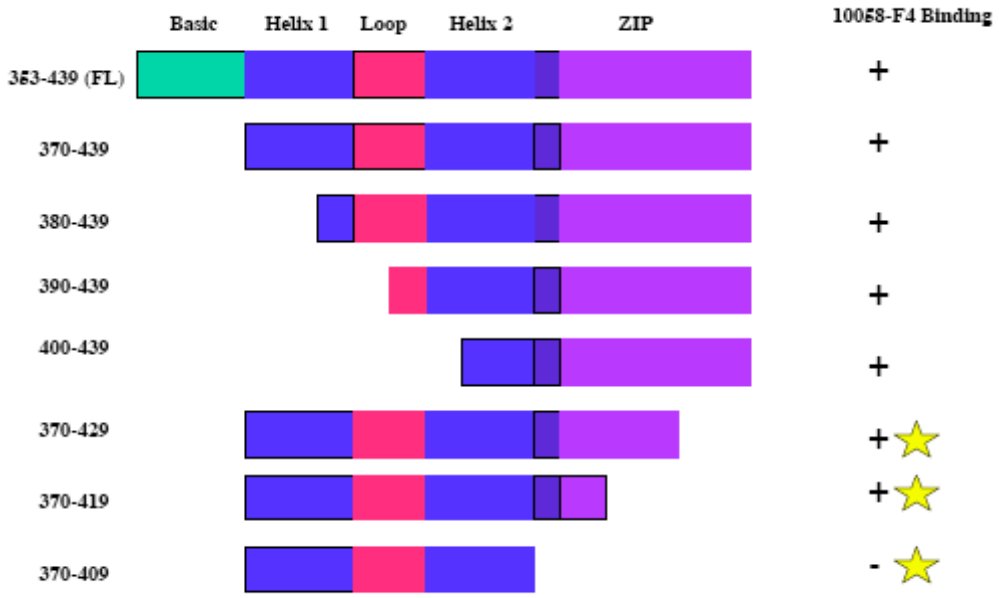

Fig. 2. Localization of binding of 10058-F4 to the c-Myc bHLH-ZIP domain. Each of the depicted deletion mutants was expressed in E. coli as a His6 fusion in the pTOPO-pET3b vector (Invitrogen). The proteins were purified to homogeneity by Ni^{+}-agarose affinity chromatography and HPLC following TEV-protease mediated cleavage of the His6 tag. Fluorescence polarization profiles were then determined as described in Fig. 1. No partial binding was observed. Stars indicate the deletions most important in narrowing down the binding sites for the respective compounds. FL: the full-length 86 amino acid c-Myc bHLH-ZIP domain, which is necessary and sufficient for Max heterodimerization.b

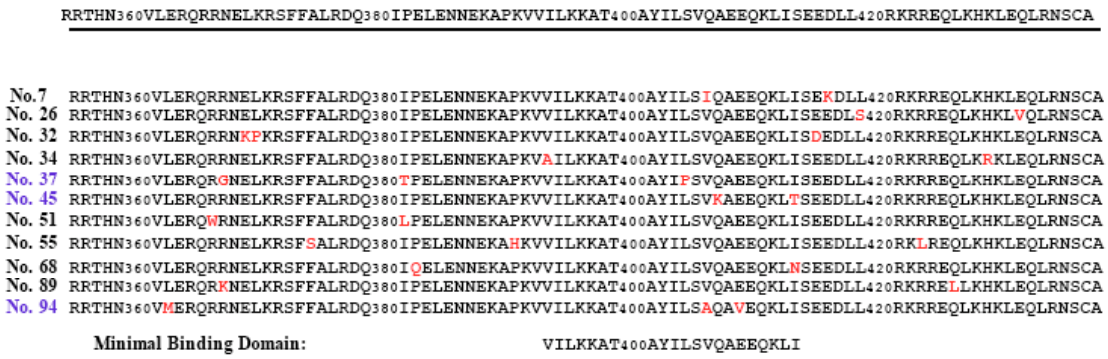

Fig. 3. Binding of 10058-F4 to c-Myc bHLH-ZIP peptides containing point mutations. The underlined sequence at the top of the diagram depicts the wild-type human c-Myc bHLH-ZIP domain. Based on the findings shown in Fig. 2, mutations were selected so as to be biased for those falling within the suspected site of 10058-F4 binding. Others were chosen to fall near, but not within, the site. In most cases, double and triple mutations were selected as they potentially provide a richer source of binding information. Amino acids highlighted in red depict the point mutations present in each mutant. Numbers in blue indicate mutants that failed to bind 10058-F4 in a standard fluorescence polarization assay (Fig.1). All other mutants, as well as the wild-type peptide, bound 10058-F4 equally well. The sequence at the bottom depicts the inferred minimal site of binding based on the above studies and is consistent with the deletion studies shown in Fig. 1.

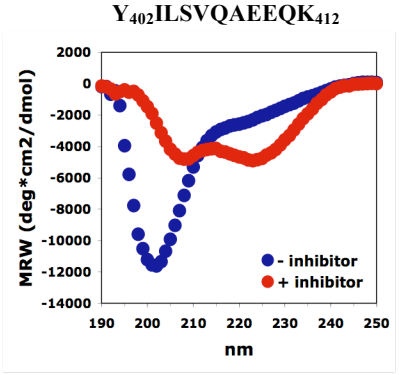

Fig. 4. Circular dichroism (CD) study of 10058-F4 bound to amino acids 402-412 of the c-Myc bHLH-ZIP domain.

B. Task 2: To conduct a series of *in vivo* studies aimed as determining whether these compounds can be effectively employed to treat c-Myc overexpressing tumors.

1. Identification of 10058-F4 analogs that are more potent than the parental compound.

Our ultimate goal is to identify analogs of the original seven c-Myc-Max compounds (REF. 2) that have greatly increased potency. We have already created a number of these based on the structure of compound no. 10058-F4. These novel analogs were generated by deliberately modifying specific sites of the 10058-F4 structure and testing them in a variety of assays ranging from the 2-component fluorescence polarization assay described in Fig. 1 to cell-based assays (REF. 3 and attached re-print). Although we identified several modifications that improved potency by as much as 8-10-fold, we believe that these improvements are still not adequate for in vivo testing. A more rational, structure-based design approach utilizing 3D NMR-determined solution structures of compounds in association with their cognate c-Myc binding sites (see below) is likely to provide better rationale for the design of newer analogs with much greater potency than we have achieved so far using a more random approach.

***C. Task 4*: To employ computerized "data mining" techniques to identify the structural properties of these compounds which impart their effectiveness.**

1. Determination of the 3D solution NMR structure of 10058-F4 in association with its c-Myc binding site.

In collaboration with Dr. Steven Metallo (Dept. of Chemistry, Georgetown Univ.), we have now solved the 3D solution structure of 10058-F4 in association with the Y402ILSVQAEEQK412 peptide (Fig. 5). This structure should provide a framework that we can now use in computational searches for 10058-F4 analogs, a project that is currently just beginning is collaboration with Dr. Ivet Behar (Dept. of Computational Biology, University of Pittsburgh). Moreover, these results have set the stage for further binding studies and NMR structures that are planned for the remaining 6 parental compounds (2). Together, this approach promises to permit the screening of literally hundreds of thousands of pre-existing compounds, so as to allow for the identification of those which best interact with their cognate binding site on the c-Myc bHLH-ZIP domain. It is our eventual goal to use these approaches to develop effective chemotherapeutic agents that can be used to treat the large number of human cancers that over-express the c-Myc oncoprotein (4).

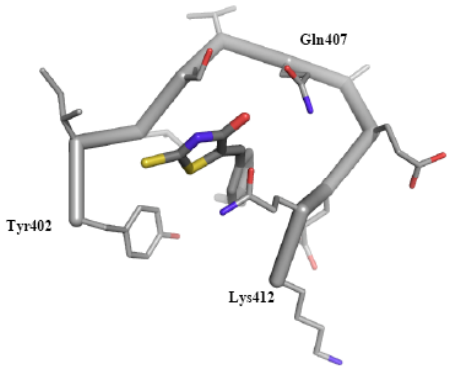

Fig. 5. The 3D NMR-derived structure of 10058-F4 in association with c-Myc.

2. Determination of the 3D solution structures of the remaining c-Myc compounds.

We originally identified seven compounds that could prevent the c-Myc-Max association (2). Using the techniques described in Figs. 2-4 above, we have now determined the binding sites for the remaining six parental compounds. Although these results are in various stages of completion we are currently able to make the following statements:

a. All compounds bind to monomeric c-Myc.
b. Compound 10074-G5 (REF. 2) binds to a distinct site of c-Myc that spans the junction of the DNA binding basic and the helix 1 domain (Fig. 2).
c. Compound 10074-A4 binds to a third site in c-Myc that is between the binding sites for 10058-F4 and 10074-G5.
d. The remaining compounds (1009-G9, 10031-B8, 10050-C10, and 10075-G5) bind to one of the previously identified sites.
e. We have nearly completed the solution 3D NMR structure of 10074-G5 in association with its minimal peptide and the structure for 10074-A4 currently being determined.

CONCLUSIONS

The initial identification of compounds that inhibit the activity of c-Myc in vitro and in vivo (2) has progressed to the point where we are now able to state that all compounds work via common mechanisms involving their direct binding to the dimerization domain of the c-Myc monomer. We know that the seven originally identified compounds bind to one of three distinct and separable sites within this domain. In three cases, 3D structures have been completed or are nearing completion. Moreover, structure-based design studies, while not showing dramatic improvements in compound efficacy have at least shown that increases in efficacy are possible. These findings support the argument that more refined analogs, based on the above 3D structures, should be much more potent that previous analogs. Moreover, because the c-Myc monomer is relatively unstructured, we believe that knowledge of the different 3D structures should allow us to chemically link two optimized structures so as to allow for the simultaneous and synergistic targeting of two distinct binding sites.

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Improved low molecular weight Myc-Max inhibitors

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Abstract

Compounds that selectively prevent or disrupt the association between the c-Myc oncoprotein and its obligate heterodimeric partner Max (Myc-Max compounds) have been identified previously by high-throughput screening of chemical libraries. Although these agents specifically inhibit the growth of c-Myc–expressing cells, their clinical applicability is limited by their low potency. We describe here several chemical modifications of one of these original compounds, 10058-F4, which result in significant improvements in efficacy. Compared with the parent structure, these analogues show enhanced growth inhibition of c-Myc–expressing cells in a manner that generally correlates with their ability to disrupt c-Myc-Max association and DNA binding. Furthermore, we show by use of a sensitive fluorescence polarization assay that both 10058-F4 and its active analogues bind specifically to monomeric c-Myc. These studies show that improved Myc-Max compounds can be generated by a directed approach involving deliberate modification of an index compound. They further show that the compounds specifically target c-Myc, which exists in a dynamic and relatively unstructured state with only partial and transient α-helical content. [Mol Cancer Ther 2007;6(9):1–10]

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Introduction

Over the last several years, numerous approaches have been used to inhibit the expression or function of the c-Myc oncoprotein, which is frequently overexpressed in human cancers (1–3). Through its role as a general basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor, c-Myc regulates hundreds of downstream target genes. The products of many of these promote transformation and control other aspects of the ''c-Myc phenotype'' by virtue of their effects on growth, metabolism, proliferation, apoptosis, and differentiation (4–6). Efficient and selective inhibition of c-Myc is thus a major therapeutic goal. Among the direct approaches taken to inhibit c-Myc have been the use of triplex-forming oligonucleotides, which interfere with *CMYC* gene transcription, and antisense oligonucleotides, which either promote c-Myc mRNA degradation or inhibit its translation (7, 8). Indirect approaches have included the specific inhibition of downstream *c-Myc* target genes (9, 10) and ''suicide'' vectors encoding cytotoxic proteins under the control of c-Myc–responsive promoters (11). Despite some successes, most of these approaches continue to be hampered by technical difficulties pertaining largely to delivery and the fact that many transforming *c-Myc* target genes are functionally redundant and/or cell type specific (2).

More recently, we and others have used a different approach that uses low molecular weight compounds (hereafter referred to as ''Myc-Max compounds'') to inhibit or reverse the association between c-Myc and its obligate bHLH-LZ heterodimerization partner, Max (12, 13). In their transcriptionally active form, c-Myc and Max not only dimerize with one another but also form bivalent heterotetramers, thus potentially explaining how c-Myc can interact simultaneously with widely separated binding sites (E-boxes; refs. 5, 14). Negative gene regulation by c-Myc also requires Max, although DNA binding occurs at non–E-box-containing InR elements located at transcriptional initiation sites or enhancer elements often located near the proximal promoter region (6). Thus, Myc-Max compounds abrogate not only c-Myc-Max heterodimerization and DNA binding but also all subsequent downstream functions. The concurrent disruption of c-Myc-Max heterotetramerization might also interfere with target gene expression in other ways, given that this higher order structure has been proposed to serve as a platform for the recruitment of other transcription factors (14).

The major problem with all Myc-Max compounds described thus far, which limits their clinical utility, is their relatively low potencies, with significant inhibition of tumor cell growth being obtained only with concentrations in the range of 50 to 100 μmol/L. We have therefore attempted in the current work to use a directed chemical design approach as a means of identifying novel analogues with improved efficacies.

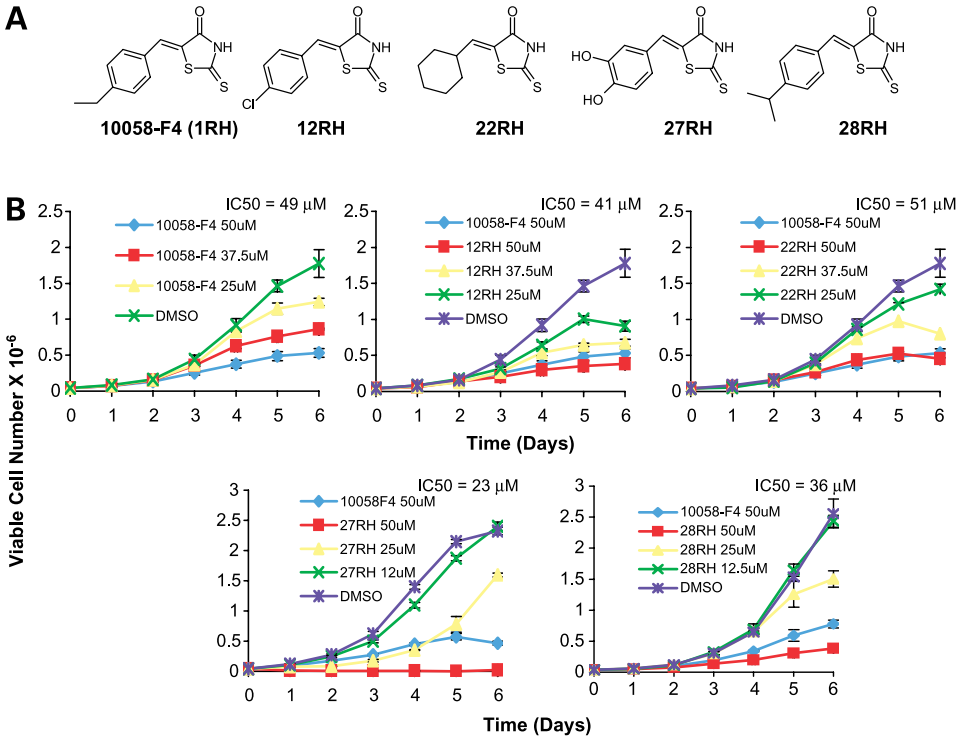


Figure 1. A, structures of the 10058-F4 (1RH) index compound and the five most potent six-member ring analogues (also see Supplementary Fig. S1). Note that all structures shown here and in Figs. 2 and 3 have been drawn in their Z-isoforms and some bonds have been standardized. **B,** dose-response profiles of each of the compounds on HL60 cell growth. IC_{50}s here, as well as in Figs. 2 and 3, were calculated based on dose-response profiles on day 5 following the addition of each compound. Representative experiments are shown, with each compound being assayed in separate experiments on two to four additional occasions. Note that cell viability at all points tested exceeded 80%. As reported previously (13), most of the compounds caused a G_0-G_1 growth arrest.

The starting compound for the current studies, (Z,E)-5-(4-ethylbenzylidene)-2-thioxothiazolidin-4-one (hereafter referred to as 10058-F4), is one of six first identified by our group and is structurally the simplest, being composed of a six-member ethylbenzylidine ring and a five-member thioxothiazolidin-4-one, or rhodanine ring (Fig. 1A; ref. 13). The ability of 10058-F4 to target c-Myc-Max, to disrupt the heterodimer and/or to prevent its formation, and to abrogate various c-Myc–dependent functions has also been confirmed independently by several groups other than our own (15–18). In combination, these properties have established10058-F4 as an attractive starting point for the generation of analogues with improved efficacy.

We describe here the consequences of altering both the six-member ethylbenzylidine ring and the five-member rhodanine ring of 10058-F4, either individually or in combination. Using several independent assays, we have identified several single ring derivatives with superiority to 10058-F4. Our results provide proof of principle that improved Myc-Max compounds can be obtained by a stepwise design approach. In addition, our finding that 10058-F4 and its active analogues bind specifically to monomeric c-Myc has implications for the future design of even more potent compounds.

Materials and Methods

In silico Screening for Myc-Max Compounds

To search for compounds related to 10058-F4 and which varied only in the six-member ring, we used the ChemFinder 7.0 software program (CambridgeSoft). The 10058-F4 structure was then used to search the structure database file for the ChemDiversity library set (5,040 total compounds, ChemDiversity) for related compounds. To search for 10058-F4 analogues, which varied only in the five-member ring, we used a web-based search program[6] (Chembridge Corp.) to screen a total of ~500,000 drug-like low molecular weight molecule compounds from the company's library with a substructure and similarity of >85%. A total of 141 compounds were identified by this method and 11 were chosen for more in-depth study.

Synthesis of 10058-F4 Analogues

Detailed protocols describing the synthesis of all 10058-F4 analogues are provided as Supplementary Data.[7] All compounds were ≥95% pure (as detected by ^{1}H nuclear magnetic resonance). All compounds also displayed solubilities ≥50 μmol/L in 1 × PBS buffer (pH 7.3). Solubilities were further assessed up to 200 μmol/L [the highest concentrations used in electrophoretic mobility shift assay (EMSA)] for the following compounds: 10058-F4, 12RH, 22RH, 27RH, 28RH, 1RH-S-Me, 1RH-NCN-1, #015, #474, #764, 12RH-NCN-1, and 28RH-NCN-1. A pH dependence was observed for the solubility of parent compound 10058-F4: in acidic pH (MES buffer; pH 5.3), its saturation concentration drops to ~50 μmol/L.

[6] https://www.hit2lead.com/search_sc.asp
[7] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Growth of Mammalian Cells

HL60 human promyelocytic leukemia cells were grown in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (all from Mediatech, Inc.). Rat fibroblast lines were grown under similar conditions in Dulbecco's modified MEM. To determine the effects of Myc-Max compounds on HL60 cell growth, logarithmically growing cells (>90% viability) were resuspended in fresh medium. Four milliliters (a total of 16,000 cells) were then seeded into six-well plates in the presence of the indicated amount of Myc-Max compound. In all cases, 10058-F4 was included as a reference compound. Daily cell counts were done manually in triplicate on a hemacytometer using trypan blue exclusion. Viabilities exceeded 85% throughout the course of the experiment. Each experiment was repeated at least two additional times with results similar to those depicted here obtained.

Preparation of Cell Lysates and Coimmunoprecipitation Experiments

Nuclei from HL60 cells were prepared essentially as described previously (17). Briefly, $\sim 4 \times 10^7$ washed nuclei were resuspended in 1 mL ice-cold buffer F, which contained 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.1), 30 mmol/L sodium pyrophosphate, 5 μmol/L $ZnCl_2$, 0.1% NP40, 0.1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, and 2.5 units/mL each of pepstatin, leupeptin, and aprotinin. All reagents were from Sigma-Aldrich. The nuclear suspension was then disrupted with the microtip of a Branson sonifier at a setting of 5 for 60 s, clarified by centrifugation (10,000 × *g* for 10 min), and stored in 200 μL aliquots at −80°C. Aliquots were thawed only a single time for use in immunoprecipitations.

To do immunoprecipitations, a total of 200 μL of the above-described nuclear extract was diluted in 0.5 mL buffer F along with the indicated final concentration of Myc-Max compound. Following incubation at 30°C for 30 min, a polyclonal rabbit anti-Max antibody (19) was added to a final dilution of 1:250 and the mixture was incubated with constant agitation at 4°C for 16 h. Protein G-Agarose (20 μL; Santa Cruz Biotechnology, Inc.) was then added for an additional 6 h with agitation. The precipitate was collected by centrifugation, washed thrice in buffer F, and boiled in SDS-PAGE lysis buffer. Western blotting of the lysate was then done as described previously (19). The upper portion of the blot was probed overnight with a 1:1,000 dilution of a murine anti-c-Myc monoclonal antibody (mAb) 9E10 (Santa Cruz Biotechnology) and the lower portion was probed with a 1:1,000 dilution of the H-2 murine mAb against Max (Santa Cruz Biotechnology). In both cases, the blots were then subsequently probed with a 1:10,000 dilution of horseradish peroxidase–conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity, Pierce) according to the directions of the supplier.

Expression and Purification of Recombinant c-Myc$_{353-439}$ and Max

The expression vector c-Myc/pET SKB3 (encoding the hexahistidine-tagged bHLH-LZ region of human Myc

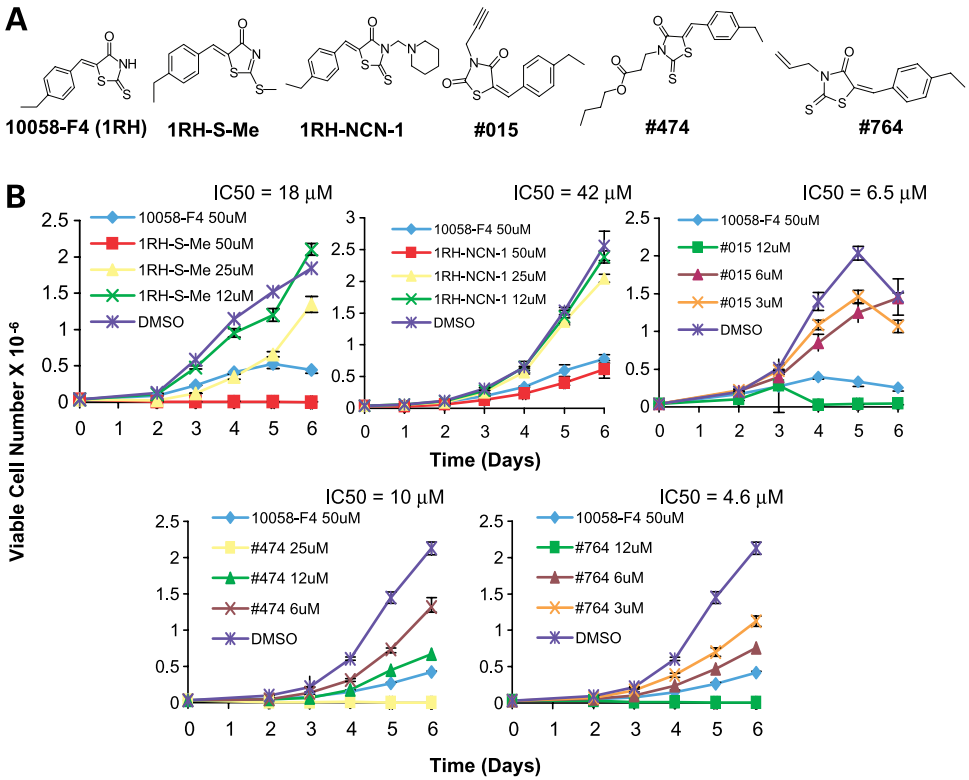


Figure 2. **A,** structures of the most active five-member ring analogues (also see Supplementary Fig. S2). Note that all compounds bearing the ''1RH'' prefix contain a six-member ring identical to that in 10058-F4. **B,** dose-response profiles of each of the compounds on HL60 cell growth.

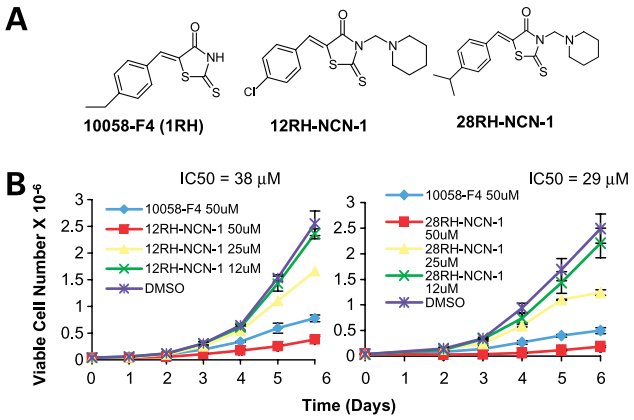


Figure 3. **A,** structures of hybrid compounds containing ''optimized'' six- and five-member rings derived from select compounds shown in Figs. 1A and 2A, respectively. **B,** HL60 proliferation assays done with the above compounds.

residues 353 to 439 with a GGCD extension at the COOH terminus) was kindly supplied by Dr. S.K. Nair (University of Illinois, Urbana-Champaign, Urbana, IL) and overexpressed in *Escherichia coli* strain BL21DE3(plysS). Hexahistidine-tagged human Max isoforms, Max(L) (160 amino acids) and Max(S) (151 amino acids), both in the pQE-10 vector (Qiagen; refs. 19, 20), were overexpressed in *E. coli* strain M15(pRep4). Briefly, bacterial cultures were grown at 37°C in LB to an $A_{600} \approx 0.8$ and then induced with 0.5 mmol/L isopropyl-L-thio-B-D-galactopyranoside for 5 h. Cultures were harvested and lysed in a buffer containing 8 mol/L urea, 100 mmol/L NaH_2PO_4, and 10 mmol/L Tris (pH 8.0). Proteins were purified on an NTA-Ni column with a pH gradient elution. Max proteins were further purified by reversed-phase high-pressure liquid chromatography. The hexahistidine tag of c-Myc$_{353\text{-}439}$ was cleaved using TEV protease [expressed previously in a pET24 vector (from Dr. S.K. Nair) and purified on NTA-Ni-agarose under native conditions]. The final c-Myc bHLH-LZ product was then further purified by high-pressure liquid chromatography and lyophilized.

Electrophoretic Mobility Shift Assays

Experiments were done on 8% polyacrylamide/bis-acrylamide (80:1) gels in 0.5 × Tris-borate EDTA. Binding reactions were prepared in a buffer consisting of 1 × PBS (pH 7.3), 1 mmol/L EDTA, 0.1% NP40, 5% glycerol, 1 mmol/L DTT, and 0.4 mg/mL bovine serum albumin. A 22 bp E-box–containing dsDNA oligonucleotide labeled on one strand with hexachlorofluoresceine consisted of the sequence 5′-hexachlorofluoresceine-CACCCGGT-*CACGTG*GCCTACAC-3′and was synthesized by Integrated DNA Technologies, Inc. The oligonucleotide was used at 10 nmol/L concentration in all reactions, which also contained 60 nmol/L each of purified c-Myc bHLH-LZ, Max(S), and the indicated amount of each compound. Proteins were first incubated for 90 min at 25°C, followed by addition of the oligonucleotide and an additional 15 min of incubation before loading on a running gel. Gels were run at 20°C and scanned on a Bio-Rad FX molecular imager (Bio-Rad). Data were analyzed with Bio-Rad Quantity One software.

Fluorescence Polarization Assays

Samples of inhibitor at 25 μmol/L concentration, in the absence and presence of an equimolar concentration of purified c-Myc$_{353\text{-}439}$ peptide, were prepared in 1 × PBS buffer (pH 7.4), 1 mmol/L DTT, and 5% DMSO. The samples were analyzed in a Photon Technology International QuantaMaster fluorimeter equipped with polymer sheet polarizers at an excitation wavelength of 380 nm and an emission wavelength of 468 nm. Alternate settings (excitation, 470 nm; emission, 600 nm) were used for the compounds 7RH and 8RH, which have longer wavelength absorption and emission spectra. Each sample was analyzed in triplicate at 25°C with sample-specific G-factor determination. Titration experiments were done with the same instrumental settings, temperature, and buffer conditions on 2-fold serial dilution of equimolar mixtures of inhibitor and c-Myc$_{353\text{-}439}$. Reported data represent the average of three to five independent experiments. Data were fit to a quadratic equation derived from the thermodynamic expression of binding equilibrium:

$$\frac{[\text{complex}]}{[C]_0} = \frac{2 + K_{\text{obs}}/[C]_0 - \sqrt{(-2 - K_{\text{obs}}/[C]_0)^2 - 4}}{2} \quad \text{(A)}$$

where $[C]_0$ represents the total concentration of inhibitor and of c-Myc$_{353\text{-}439}$. The value of K_{obs} was determined from the experimental polarization data by fitting to Eq. B using KaleidaGraph (Synergy Software) where pol_0 is the polarization in the absence of binding and Δpol is the total change in polarization (21).

$$\text{polarization} = \text{pol}_0 + \Delta\text{pol}\left(\frac{[\text{complex}]}{[C]_0}\right) \quad \text{(B)}$$

Results

Modification of the Six-Member Ring of 10058-F4

To identify candidate 10058-F4 analogues with improved efficacy, we conducted an initial *in silico* screen of the 5,040 member low molecular weight ChemDiversity library for structures that shared the same five-member rhodanine ring as the parental compound but that contained variations of the six-member ring. This search yielded a total of ten so-called ''second-generation'' compounds. We also synthesized a library of 38 additional compounds. The structures of all compounds are depicted in Supplementary Fig. S1.[7]

Each compound was initially tested for its ability to inhibit the growth of the HL60 human promyelocytic leukemia cell line, which expresses high levels of c-Myc as a result of gene amplification (21). Because this assay is biologically based, it serves as an easy, rapid, and accurate means of eliminating pharmacologically inactive agents. In each case, 10058-F4 was included in parallel assays to

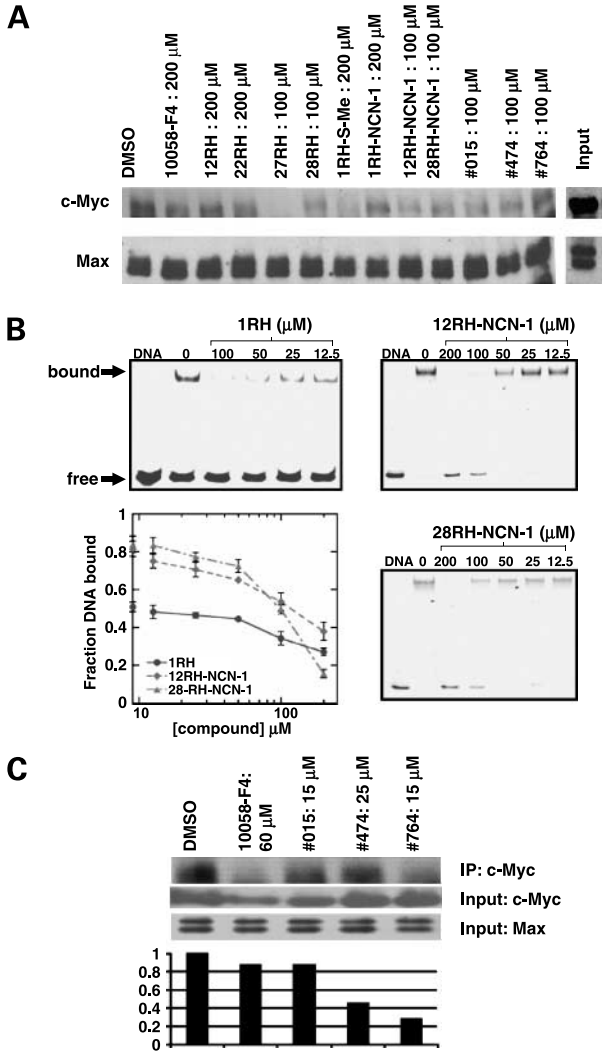


Figure 4. **A,** coimmunoprecipitation of c-Myc and Max from HL60 nuclear extracts. Equivalent amounts of nuclear extracts were incubated with the indicated concentrations of the compounds depicted in Figs. 1 to 3. Following precipitation of the complexes with an anti-Max antibody, the total amount of associated c-Myc was detected by immunoblotting (*top*). As a control, the lower portion of the blot was probed with an anti-Max antibody (*bottom*). *Extreme right hand column,* the total input of c-Myc and Max proteins before immunoprecipitation. **B,** EMSA results and quantitation. Recombinant c-Myc$_{353-439}$ and full-length Max(S) were purified to homogeneity from *E. coli* and used at a final concentration of 60 nmol/L in the presence of the indicated concentration of compound. Each experiment was done at least thrice with typical results shown here. The graph summarizes quantitative analyses for the depicted compounds from three independent assays. See Supplementary Fig. S4 for EMSAs with additional active analogues. Control experiments showed that none of the compounds significantly affected DNA binding by Max(L) homodimers (Supplementary Fig. S5). **C,** disruption of c-Myc-Max heterodimers *in vivo*. HL60 cells (5×10^6) were treated for 4 h with the indicated concentrations of 10058-F4 or three of its analogues in 5 mL of medium lacking serum. Cell lysates were then prepared and assayed for total c-Myc and Max levels (*Input*). The remainder of the lysate was used for coimmunoprecipitation experiments as described in (**A**). Note the reduced levels of endogenous c-Myc in total lysates from10058-F4 – treated cells. The histogram was derived by densitometric scanning and depicts the ratios of total c-Myc in immunoprecipitation (*IP*) experiments compared with total lysates. The ratio between these levels in control cells was arbitrarily set at 1.

permit direct and immediate comparison with all test compounds. From this initial screen, we identified, as expected, several analogues that were at least as potent as 10058-F4. Figure 1 shows the structures of the four most active analogues, their dose-response profiles, and their IC_{50}s. From these results, it can be seen that one compound, namely 27RH, was approximately twice as potent as 10058-F4 (IC_{50}, 23 versus 51 μmol/L), whereas a second compound, 28RH, was only marginally better (IC_{50}, 36 μmol/L). Although profoundly growth inhibited at higher compound concentrations, the cells in all cases remained >80% viable after 4 to 5 days as determined by trypan blue staining (data not shown). In addition and as reported previously (13), cell cycle analysis showed that exposure to these compounds resulted in growth arrest in the G_0-G_1 stage of the cell cycle (data not shown). We conclude that alterations of the six-member ring of the 10058-F4 index compound lead to modest but significant improvements of *in vivo* activity with growth inhibitory. The frequency with which compounds are identified that are more potent than 10058-F4 is also quite low.

Modification of the Five-Member Rhodanine Ring of 10058-F4

The foregoing studies established that the structure of six-member ring of 10058-F4 could be altered so as to produce analogues with modestly improved efficacy. To explore further the consequences of other structural alterations, we did an additional *in silico* screen of a 500,000-member low molecular weight compound library (Chembridge) for 10058-F4 analogues whose only modification was in the five-member rhodanine ring. A total of 11 compounds were identified in this way and four additional ones were synthesized. The structures of each of these compounds are depicted in Supplementary Fig. S2,[7] and those of the five most active compounds are depicted in Fig. 2A.

Each analogue was again tested in HL60 cells as described above. Although numerous active compounds were identified, only four (1RH-S-Me, #015, #474, and #764) were significantly more active than 10058-F4 with IC_{50}s ranging from 4.6 to 18 μmol/L (Fig. 2B).

Combined Modification of the Five- and Six-Member Rings of 10058-F4 Fail to Provide Additive Potency

The foregoing results indicated that certain modifications of the component rings of 10058-F4 lead to enhanced activity. To determine whether the idealized ring structures from these ''second-generation'' compounds could be combined additively so as to further improve their activities, we next synthesized and tested a group of 17 ''third-generation'' compounds containing select combinations of optimized five- and six-member rings (Fig. 3A; Supplementary Fig. S3).[7] The choice of each starting ring structure was based on a combination of the results of screens depicted in Figs. 1 and 2, preliminary evaluation in EMSAs (data not shown), and the ease of synthesis and yield of the final compound. As shown in Fig. 3B and C, only two compounds (i.e., 12RH-NCN-1 and 28RH-NCN-1) showed activities comparable with that of 10058-F4 in HL60 cells. Of particular significance was that each of

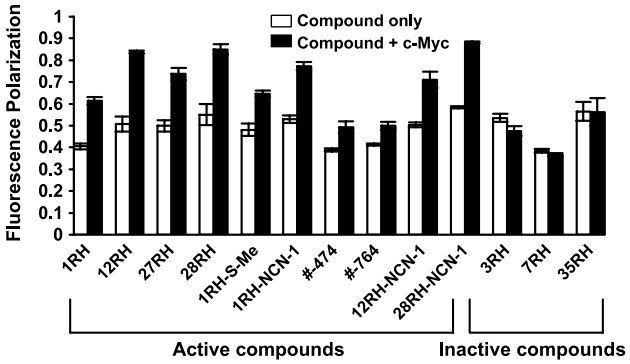


Figure 5. Fluorescence polarization assays. Each of the indicated compounds was assayed in triplicate at a final concentration of 25 μmol/L in the presence or absence of 25 μmol/L recombinant c-$Myc_{353-439}$. Excitation and emission maxima were 380 and 468 nmol/L, respectively.

these was either inferior to or only marginally better than each of its second-generation predecessors. From these and the foregoing studies, we conclude that the greatest improvements in efficacy resulted from select changes in only the rhodanine ring of 10058-F4.

10058-F4 Analogues Interfere Directly with c-Myc-Max Heterodimerization and DNA Binding

The above studies were designed to serve as rapid, biologically based screens for Myc-Max compounds with the greatest *in vivo* potencies. However, they did not necessarily establish that the observed effects were due specifically to the disruption of c-Myc-Max complexes, as had been shown previously for the parent 10058-F4 compound (13). To address this, we determined the effect of each compound on c-Myc-Max association *in vitro* by two different methods. In the first, nuclear extracts from HL60 cells were incubated with each compound and a coimmunoprecipitation was done with an anti-Max antibody (19). The total amount of coprecipitating c-Myc protein was then assessed by immunoblotting. As a coimmunoprecipitation control, the same blot was also probed for Max. As shown in Fig. 4A, 10058-F4, as well as all tested analogues, promoted the dissociation of c-Myc from Max in this assay. In general, good, albeit inexact, correlations between this assay and *in vivo* assays were observed. Control experiments further established that none of the compounds affected the absolute levels of either c-Myc or Max (Fig. 4A; data not shown).

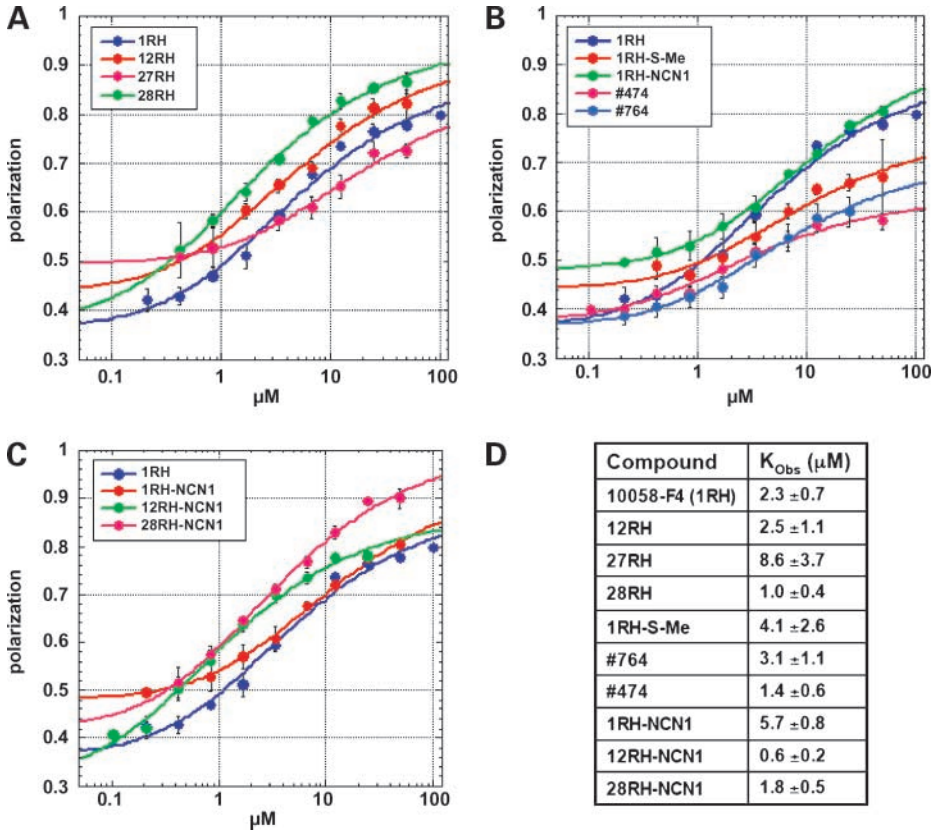
The second method used to gauge the effects of 10058-F4 and its analogues on c-Myc-Max complexes relied on the use of a three-component EMSA. For this purpose, a recombinant c-Myc peptide, which encompasses the bHLH-LZ domain (c-$Myc_{353-439}$), together with the full-length hexahistidine-tagged 151–amino acid isoform of Max [designated Max(S); refs. 19, 20] were incubated with increasing concentrations of each relevant compound. The ability of the resultant heterodimer to bind a double-stranded target oligonucleotide containing a consensus E-box motif was then assessed by PAGE. To simplify interpretation of the assay, we purposely used Max(S) because, unlike the160–amino acid isoform, Max(L), it is unable to bind DNA as a homodimer at the concentrations used here (19, 20). Because c-Myc is also unable to form homodimers, any observed shifted band must be indicative of DNA binding by the c-Myc-Max(S) heterodimer (19, 20). As seen in Fig. 4B and Supplementary Fig. S4,[7] DNA binding was readily observable in the absence of any added compound, whereas the addition of 10058-F4 or its analogues resulted in a dose-dependent, although variable, inhibition, with compounds 28RH and 12RH-NCN1 being among the most effective. In control experiments (Supplementary Fig. S5),[7] we have shown that DNA binding by the Max(L) homodimer was unaffected by these compounds. As for the coimmunoprecipitation experiments, these studies establish a correlation between the *in vivo* efficacy of certain of these compounds and their ability to affect c-Myc-Max association and DNA binding *in vitro*. In addition, they are consistent with data obtained in HL60 cells that modification of the 10058-F4 parental backbone can enhance *in vivo* efficacy.

Having shown that 10058-F4 and several of its more potent analogues could disrupt c-Myc-Max heterodimers and inhibit their ability to bind DNA, we next asked whether similar effects could be observed *in vivo*. HL60 cells were therefore incubated with each compound for 4 h, and c-Myc-Max complexes were immunoprecipitated as described above. As shown in Fig. 4C and as described previously (18), 10058-F4 caused a reduction in total c-Myc levels. When this was taken into account, the compound promoted an ~20% reduction in the remaining c-Myc-Max complexes at the concentration tested (60 μmol/L). In marked contrast, the three analogues inhibited c-Myc-Max association by 20% to 75% at significantly lower concentrations (15–25 μmol/L) and had no effect on total c-Myc levels.

10058-F4 and Its Analogues Bind Directly to c-Myc

Previous studies with 10058-F4 and other structurally unrelated index compounds had not specifically addressed the question of whether their binding required intact c-Myc-Max heterodimers or could occur on monomeric forms of the proteins. To investigate this, we took advantage of the fact that 10058-F4, and most of its analogues, are fluorescent and can depolarize an incident beam of light. Because the degree to which this occurs is partly a function of the rate of tumbling of molecule in solution, binding to either c-Myc or Max should result in a loss of fluorescence depolarization. As seen in Fig. 5, this occurred for 10058-F4 and all active analogues on the addition of c-$Myc_{353-439}$. In contrast, inactive 10058-F4 analogues failed to bind (Fig. 5).[8] Similar experiments done with recombinant Max(S) protein failed to provide evidence for binding of any of the compounds (data not shown). Together with our other findings, these results support the idea that the activities of 10058-F4 and its

[8] Unpublished data.



Compound	K_{Obs} (μM)
10058-F4 (1RH)	2.3 ±0.7
12RH	2.5 ±1.1
27RH	8.6 ±3.7
28RH	1.0 ±0.4
1RH-S-Me	4.1 ±2.6
#764	3.1 ±1.1
#474	1.4 ±0.6
1RH-NCN1	5.7 ±0.8
12RH-NCN1	0.6 ±0.2
28RH-NCN1	1.8 ±0.5

Figure 6. Fluorescence titration assays. Samples of inhibitor at 2-fold dilutions, both in the absence and presence of c-Myc$_{353-439}$, were analyzed as described in Fig. 5 and in Materials and Methods. **A,** titrations for six-member ring-substituted compounds 12RH, 27RH, and 28RH. **B,** titrations for rhodanine ring-substituted compounds 1RH-S-Me, #474, and #764. **C,** titrations for third-generation, dual-substituted compounds 1RH-NCN1, 12RH-NCN1, and 28RH-NCN1. **D,** calculated K_{obs} values based on the above titration profiles. Note that in all cases, the index compound 10058-F4 (1RH) was used as a control. Also note that compound #015 was not included in these and other fluorescence polarization assays due to its lack of fluorescence.

analogues arise from their ability to bind directly to the c-Myc bHLH-LZ monomer.

The binding affinities for selected active compounds were determined by titrating them with c-Myc$_{353-439}$ and following the change in polarization of their intrinsic fluorescence. When unbound, the inhibitors exhibit low fluorescence polarization. When excited with polarized light, they emit substantially depolarized light due to their rotation during the fluorescence lifetime. When the compounds are bound to c-Myc$_{353-439}$, the polarization increases due to slower tumbling and this change can be used to calculate an observed binding constant. The affinity of parental 10058-F4 for c-Myc$_{353-439}$ determined this way was 2.3 ± 0.7 μmol/L (Fig. 6). In the initial set of modifications to the aromatic moiety, 12RH was found to have an affinity similar to 10058-F4, whereas the dihydroxy derivative 27RH was severalfold worse in this direct binding assay. In this group, only 28RH (1.0 ± 0.4 μmol/L) was found to bind better than 10058-F4 (22RH is nonfluorescent and could not be assayed). With the exception of the linear ester derivative (#474), modification of the rhodanine ring lead to an ~2-fold decreases in c-Myc$_{353-439}$ affinity. Combining modifications that either did not change binding (12RH) or reduced binding (1RH-NCN1) relative to 10058-F4 actually led to the tightest binding compound, 12-RH-NCN1, which had an affinity of 0.6 ± 0.2 μmol/L. The nonadditive nature of the modifications may be a consequence of the flexibility of monomeric, predominately unstructured c-Myc$_{353-439}$, which may adopt somewhat different conformations to bind modified compounds. A compound with modifications at both sites may be binding a c-Myc conformation different from that which binds compounds with only singly modified rings.

10058-F4 Analogues Show Selectivity for c-Myc–Expressing Cells

Our initial mammalian cell–based screening assays for Myc-Max compound efficacy (Figs. 1–3) were done in HL60 cells because of their high level expression of c-Myc (22). We reasoned that those compounds capable of inhibiting HL60 growth should also be effective against other tumor lines, which generally express lower levels of c-Myc. However, inhibition of HL60 growth per se does not prove *in vivo* specificity, although our *in vitro* assays were consistent with such a conclusion. To test this, we evaluated several of the most potent compounds in three related Rat fibroblast cell lines. The first, HO16.4C, is a homozygous c-Myc$^{-/-}$ ''knockout'' cell line (KO cells) derived by homologous recombination from the second, parental cell line, TGR1 (23). The third cell line, KO-HMG, was derived from HO16.4C cells and ectopically expresses HMGA1b, a member of the high mobility group of architectural transcription factors. We have shown previously that KO-HMG cells grow more rapidly than TGR1 cells despite their lack of c-Myc expression (24). As seen in Fig. 7, TGR1 cells showed significant growth inhibition at the compound concentrations tested. In contrast, both KO

cells and KO-HMG cells were significantly more resistant to identical concentrations of the compounds. Because KO-HMG cells divide at least as rapidly as the TGR cell line from which they are derived (24), these differences cannot be attributed to any disparities in growth rates of the cell lines. From these studies, we conclude that, like the parental compound, the ability of 10058-F4 analogues to inhibit the growth of mammalian cells is c-Myc dependent.

Discussion

Resolution of the pathways leading to malignant transformation and progression has allowed for the rational design of chemotherapeutic agents with improved specificities and therapeutic indices (25–27). The as yet early successes with such ''targeted therapies'' make it highly likely that similar strategies will continue to be used well into the future.

CMYC is among the most commonly deregulated oncogenes in human cancer. The c-Myc oncoprotein is general bHLH-LZ transcription factor that regulates hundreds of downstream target genes (1, 2, 4–6). Consequently, c-Myc, in addition to promoting transformation, exerts control over such basic cellular properties as proliferation, growth, metabolism, and differentiation. Several model systems have clearly shown the ongoing need for c-Myc to maintain tumor growth and viability, thus underscoring its attractiveness as a therapeutic target (28, 29).

In the current work, we have extended our previous findings and those of others showing that low molecular weight compounds or short helix-1–related bHLH-LZ peptidomimetics can prevent or disrupt c-Myc-Max heterodimer formation or its binding to E-box motifs (12, 13, 17, 30, 31). A shortcoming of all low molecular weight compounds described thus far, however, has been their generally low potency, which detracts from their utility in actual clinical settings. This likely reflects their having been identified in screens of chemical libraries, whose finite contents are unlikely to contain clinically optimized structures (32). In the current study, we have attempted to rectify this by concentrating on the least structurally complex member of our original set of index compounds (i.e., 10058-F4). Our intention was to synthesize, or identify by *in silico* means, 10058-F4–related ''second-generation'' compounds with enhanced potency. We did these initial surveys in three stages. In the first, the five-member rhodanine ring of 10058-F4 was maintained while modifying the six-member ring. In the second stage, modifications of only the rhodanine ring were evaluated. Together, this population of novel compounds provided a working library of analogues, a number of which proved superior to 10058-F4. Finally, we asked whether the best of these second-generation structures could be combined to generate even more potent ''third-generation'' compounds.

Realizing that the apparent efficacy of a compound might be influenced in either direction by the nature of the assay used in its evaluation, we used five different, and largely independent, assay systems. These consisted of mammalian cell–based proliferation assays, a coimmunoprecipitation assay of c-Myc-Max complexes from nuclear extracts, an EMSA assay with highly purified c-Myc and Max proteins, a coimmunoprecipitation assay from cells, and a two-component fluorescence polarization assay to directly measure compound binding to the c-Myc bHLH-LZ domain. Because all of the compounds were structurally related to 10058-F4, it was not surprising that a large number of them showed significant activities in each of these assays.

In retrospect, it is also not surprising that the utilization of multiple assays while affirming the selectivity of our analogues nonetheless complicated their prioritization with regard to their precise *in vivo* efficacies. For example, several six-member ring-substituted analogues seemed superior to 10058-F4 in EMSA or coimmunoprecipitation assays but showed no better potency than 10058-F4 in HL60 cells. Examples of such compounds included 27RH and 28RH. Conversely, certain five-member rhodanine ring-substituted compounds, such as #015 and #764, which

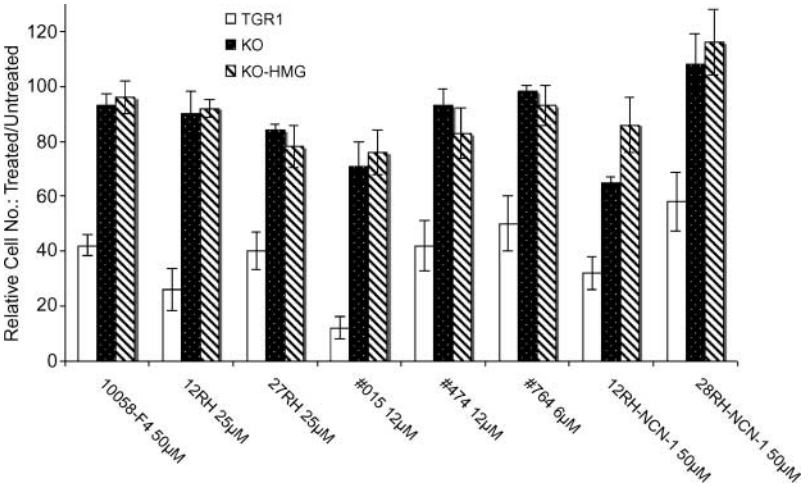


Figure 7. Specificity of Myc-Max compounds for c-Myc–expressing cells. The related rat fibroblast cell lines TGR1, KO, and KO-HMG (23, 24) were seeded at 5×10^4 cells per well into six-well plates and allowed to achieve logarithmic growth (1–2 d). Fresh medium containing the indicated concentrations of Myc-Max compounds was then added. TGR1 and KO-HMG cells were then further incubated for an additional 3 to 4 d, a point at which cells without compounds had achieved 70% to 90% confluency. KO cells were allowed to grow for an additional 4 to 5 d to compensate for their overall slower rate of proliferation, with compound-containing medium being changed every 2 to 3 d. Cells were then trypsinized and viable cell numbers were determined in triplicate cultures using trypan blue dye exclusion. All cell numbers are normalized to identical sets of control, untreated cells cultured in parallel.

showed a significantly improved antiproliferative effect against HL60 cells, did not necessarily prove superior to 10058-F4 in coimmunoprecipitation or EMSA assays. Although evaluation of the *in vivo* fates of these analogues is beyond the scope of the current work, these disparities among different assays likely reflect uncontrolled variables of cell-based assays such as compound uptake, stability, active efflux, and metabolism to more or less active analogues. Indeed, similar variability was observed between coimmunoprecipitation and EMSA assays (Fig. 4). This likely stems from the fact that the compound concentration required to disrupt a protein-dimer/DNA complex (as measured via EMSA) will be generally higher than that required to disrupt the protein-dimer alone (coimmunoprecipitation). This difference is a necessary and direct result of the differences in free energy of the complexes. In the former case, the compound is competing against (i.e., disrupting) the free energy of both protein dimerization and DNA binding, whereas, in coimmunoprecipitation studies, it is disrupting only protein dimerization. Similar disparities would be expected for any compound and any protein dimer (the DNA-dimer complex most be lower in free energy than the isolated dimer or it would simply fail to form). Finally, although 10058-F4 itself was a relatively poor disruptor of c-Myc-Max complexes (Fig. 4C), a significant aspect of its *in vivo* efficacy resulted from its destabilization of total c-Myc levels.

One important question that was addressed by the use of whole-cell assays was that of specificity. These studies revealed a significant tendency for all tested compounds to inhibit cellular growth in a highly c-Myc–dependent manner (Fig. 7). Although we cannot at this time entirely eliminate the possibility that 10058-F4 or its analogues have molecular targets other than c-Myc, the studies shown in Fig. 7 suggest that such target are likely to be relevant only at concentrations of compound exceeding the IC_{50}s established here.

With regard to fluorescence polarization measurements (Figs. 5 and 6), we note that binding for all compounds generally occurred at concentrations lower than those needed to disrupt the c-Myc-Max interaction in other assays (K_d's, 0.5–8.6 μmol/L; Fig. 6D). This likely reflects the fact that binding to monomeric c-Myc in solution occurs under conditions in which the bHLH-LZ domain exists in a mobile conformation of only partial, or transitory, α-helical content (33). Other assays, all of which involve the presence of Max, involve a competition between Max and the compound and are thus influenced by differences in the free energy of c-Myc-Max heterodimer formation. For example, EMSAs were done under conditions in which complete or nearly complete binding of the protein heterodimer to the E-box–containing oligonucleotide occurs in the absence of inhibitor. The disruption of such a protein-DNA complex at the top of its titration curve is energetically more difficult than is the disruption of the same complex under conditions of only partial binding (e.g., at or near the bottom of its titration curve). We note that some compounds (e.g., 28RH and 12RH-NCN-1) were quite effective at eliminating E-box binding by c-Myc-Max (Fig. 4B). Because the disruption observed in EMSAs is a function both of the effectiveness of compound and of the affinity of heterodimer at a particular concentration and set of binding conditions, comparison between compounds within a series is useful. However, results cannot be compared directly with those obtained under dissimilar conditions (33, 34).

An unexpected outcome of this study was that the activities of the best second-generation compounds, such as 27RH, were not substantially improved when combined with the optimized rhodanine ring derivative to create ''third-generation'' compounds (Fig. 3; data not shown). This may reflect the nature of binding to an intrinsically disordered protein where a single ''best fit'' between the compound and the target may, in fact, not exist. Thus, the protein conformation that optimizes contacts with the rhodanine ring and the substituted phenyl ring may be quite different from that which optimizes contacts with the rhodanine ring and the piperidine ring for RH-NCN1 (Fig. 3B). Binding a particular protein conformation produces an entropic penalty either from organization of certain residues into a binding conformation or from selecting one particular structure out of the ensemble of possible conformations. The current compounds may not be able to organize (or capture) a sufficiently large region of the peptide in an energetically favorably way such that the binding gains from substitutions at either end of the molecule are cumulative.

Because the number of third-generation compounds was by necessity limited, we are unable to state with any certainty that their relatively poor potencies constitute a general property. However, our findings do suggest that future improvements in compound efficacy are more likely to derive from the actual three-dimensional structure of the c-Myc bHLH-LZ in association with a compound obtained by techniques, such as nuclear magnetic resonance spectroscopy or X-ray crystallography. At a minimum, determining these structures is likely to provide help in determining which chemical groups on the low molecular weight moieties that can be modified so as to maximize and/or stabilize their interaction with the peptide. Despite the above caveats, our results do indicate that relatively simple alterations of an index compound, such as 10058-F4, can lead to significant improvements in efficacy. This provides reason to suppose that additional modifications of the structures presented here might continue to provide a source of novel compounds with improved efficacies. Their utility may perhaps be optimized even further when combined with other, unrelated low molecular weight agents that independently target other regions of the c-Myc bHLH-LZ domain.

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